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Minireview

Genome-wide application of RNAi to the discovery of potential drug targets

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Abstract Progress is being made in the development of RNA interference-based (RNAi-based) strategies for the control of gene expression. It has been demonstrated that small interfering RNAs (siRNAs) can silence the expression of target genes in a sequence-specific manner in mammalian cells. Various groups, including our own, have developed systems for vector-mediated specific RNAi. Vector-based siRNA- (or shRNA) expression libraries directed against the entire human genome and siRNA libraries based on chemically synthesized oligonucleotides now allow the rapid identification of functional genes and potential drug targets. Use of such libraries will enhance our understanding of numerous biological phenomena and contribute to the rational design of drugs against heritable, infectious and malignant diseases.

Keywords: RNA interference; small interfering RNA; Vector-based siRNA

1. Introduction

The sequence of human genome has been determined but the functions of many genes remain unknown. Methods for bridging the gap between sequence and function are obviously necessary. Various methods have been used in attempts to suppress gene expression, exploiting, for example, antisense oligonucleotides and ribozymes. Recently, RNA interference-based (RNAi-based) strategies have been developed and show considerable promise. RNAi is an evolutionarily conserved biological phenomenon in plants and animals whereby double-stranded RNA (dsRNA) induces the sequence-specific degradation of cognate RNA [1]. This process is recognized as a mechanism in defense of the genome against molecular parasites such as viruses and transposable genetic elements (transposons) [2]. It is now well established that some gene regulation is mediated by small RNAs of 19–28 nucleotides (nt) in length, such as microRNAs (miRNAs) [3] and small interfering RNAs (siRNAs) [4], generated from precursor dsRNAs by ribonuclease III type enzyme Dicer. By accumulation of many researches, RNA-mediated gene silencing has now been developed from only biological phenomenon into very useful experimental tool to suppress gene expression by miRNAs and/or more popular dsRNAs such as expressed dsRNAs and synthetic siRNAs. This review summarizes progress to date in optimizing RNAi and exploiting its potential.

In RNAi, a long dsRNA is processed intracellularly by Dicer to yield siRNAs. Human Dicer has been cloned and its ribonucleolytic activity and dsRNA-binding properties have been characterized. Recombinant Dicer generates 21- to 23-nt products from dsRNA [5,6]. The experimental silencing of specific genes by RNAi in mammalian systems was hampered initially by the non-specific dsRNA-dependent inhibition of protein synthesis via the protein kinase R (PKR) pathway and the non-specific degradation of RNA that occurs upon activation of RNase L. However, it was demonstrated that synthetic 21- or 22-nt RNAs with 2-nt 3′-overhangs (siRNAs) can silence the expression of target genes without any non-specific inhibition of gene expression in cultured mammalian cells [7]. The synthetic siRNA duplex is similar to a naturally processed siRNA product from long dsRNAs both in terms of length and in terms of structure. The dominant products of processing by Dicer are duplexes of 21- and 22-nt RNAs with 2-nt 3′ overhangs, which are very effective mediators of the degradation of mRNA [7,8], although slightly longer siRNAs of approximately 27 nts have been reported to be more effective [9,10]. It has proved possible to use siRNAs to control the expression of both exogenous and endogenous genes in mammalian cells [7–10].

2. Design of effective siRNAs

Schwarz et al. [11] showed that the two strands of an siRNA duplex are not equally eligible for assembly into the RNA-induced silencing complex (RISC) and siRNA duplexes can be functionally asymmetric, with only one of two strands having the ability to trigger RNAi. Therefore, it might be preferable to design an siRNA duplex with an antisense strand that can enter RISC. It has been shown statistically that siRNAs with functional duplexes have a lower internal stability at the

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5′-antisense end than that of non-functional duplexes [12]. Reynolds et al. [13] identified some characteristics for the rational design of siRNAs for RNAi, such as a bias towards low internal stability at the 3′ terminus of the sense strand. Ui-Tei et al. reported results that were basically consistent with these findings and the following rules appear to apply to the induction of effective gene silencing in mammalian cells: (i) there should be A or U at the 5′ end of the antisense strand; (ii) there should be at least five A or U residues in the 5′-terminal one-third of the antisense strand and (iii) there should be no GC stretch of more than 9 nt in length [14]. We also showed that U at the 10th position in the sense strand (the middle nucleotide of the target) tends to be associated with strong activity [15]. Strategies for the design of effective siRNAs are becoming clearer [11–18].

Although an siRNA duplex with a bulge in its sense strand retained most of its RNAi activity, bulges in the antisense strand, when this strand formed a duplex with its target, completely abolished the ability of the duplex to induce RNAi. These observations suggested that siRNAs should be designed to be perfectly complementary to their targets [19], although our recent data suggest that some predetermined bulges within expressed short hairpin RNAs (shRNAs; Fig. 1) in the sense or antisense strand do not disturb RNAi [20]. Miller et al. [21] demonstrated the allele-specific silencing of dominant genes associated with two unrelated diseases and concluded that siRNA can be engineered to silence the expression of disease-related alleles that differ from wild-type alleles by as little as a single nucleotide. However, it has been demonstrated that siRNAs can also cross-react with targets with limited sequence similarity [22]. Sledz et al. [23] reported that, in addition to the specific gene-silencing effects of RNAi, global interferon-stimulated enhancement of gene expression can be detected in response to the intracellular stimulation by siRNAs. Kim et al. reported that short single-stranded RNAs (ssRNAs) and siRNAs synthesized by phage polymerase also induced

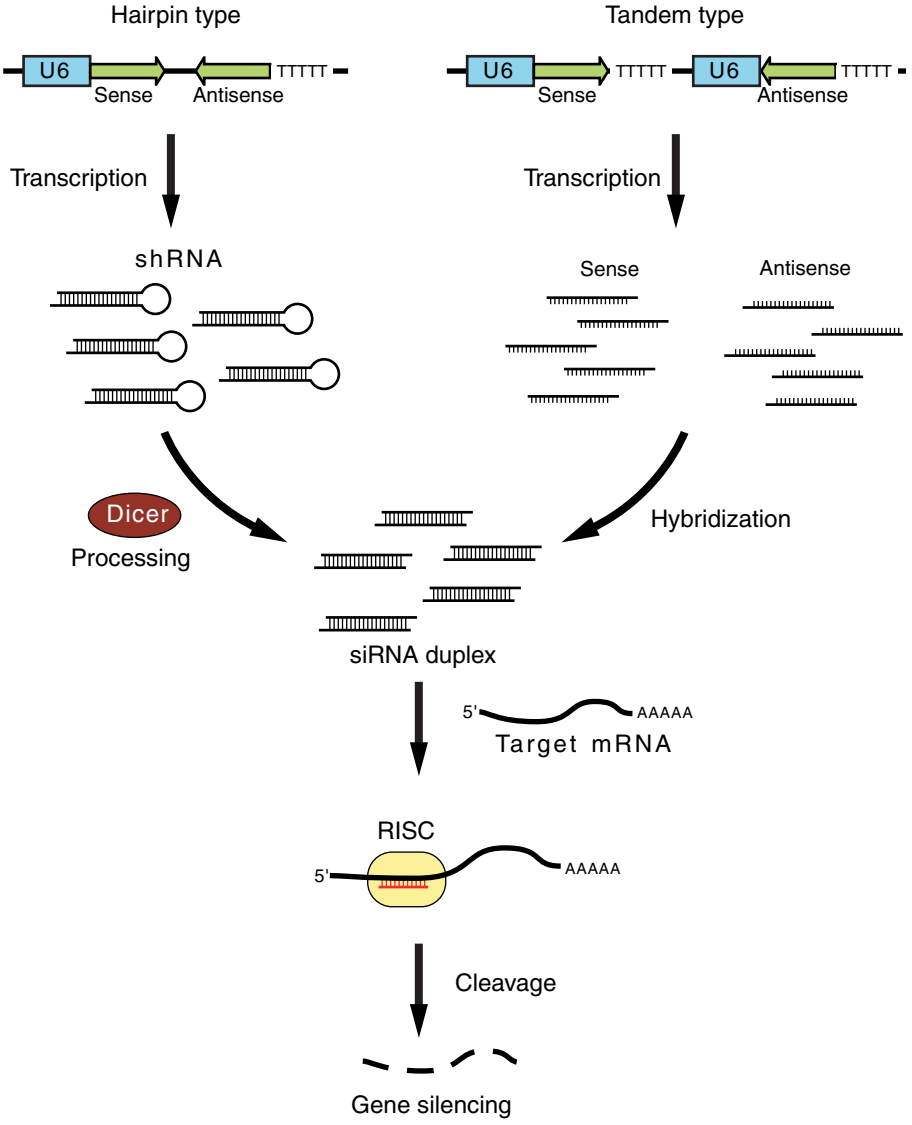

Fig. 1. Schematic representation of gene silencing by an shRNA-expression vector. The shRNA is processed by Dicer. The processed siRNA enters the RNA-induced silencing complex (RISC), where it targets mRNA for degradation.

the interferon response and that removal of the 5′ triphosphate of the transcripts prevented activation of the interferon response. Thus, their findings suggested that the interferon response could be circumvented if the 5′ triphosphate was removed from the transcribed RNA [24]. It has been reported that the interferon response can be induced by both a substantial number of shRNA vectors [25] and synthetic siRNAs with certain sequences [26,27]. Thus, use of the lowest effective dose of an shRNA vector or siRNA was recommended in order to limit the risk of the interferon response [25].

We demonstrated recently that the introduction of G:U mismatches within transcribed shRNAs significantly reduced the extent of induction of the interferon response [28,29], without any reduction in the extent of RNAi [30].

3. Systems for the expression of shRNA and genome-wide RNAi libraries

Because transfected synthetic siRNAs in cells can be degraded within a short time, inhibition of target genes does not continue for very long. To overcome this problem, various groups, including our own, have developed systems for vector-mediated specific RNAi in mammalian cells [31–39] (Fig. 1). Each of these systems exploits a polymerase III (pol III) promoter, such as a U6 or H1 promoter or the promoter of the gene for a tRNA. The various systems can be divided into two groups depending on whether the expressed RNA is of the tandem type or the hairpin type (Fig. 1). In the expression system for the tandem type, both of the sense and the antisense strand are driven separately by their own respective promoters. In the expression system for the hairpin type, a sequence that contains the sense strand of the siRNA of interest, followed by a loop sequence and the antisense strand, is driven by a single promoter. The H1 and U6 promoters have been used to drive the expression of shRNAs because they produce small RNA transcripts that lack a polyadenosine tail and have a well-defined site for initiation of transcription and a termination signal that consists of more than four T residues in a row (>T4), which results in 3′ overhanging U residues. Since it dose not contain any additional nucleotides at the 3′ end, the shRNA that is transcribed from such a vector system can function effectively. The structure of the transcribed and processed shRNA closely resembles that of synthetic double-stranded siRNA [40]. This shRNA system has been used to inhibit gene expression in mammalian cells very effectively and to an extent equal to that achieved with synthetic siRNA [41].

The genome-wide and comprehensive analysis of gene expression in various organisms should be facilitated by exploitation of the above-described phenomenon. For example, systematic functional analysis by RNAi of a number of genes in *Caenorhabditis elegans* [42–47] and *Drosophila* cells [48–52] has allowed the identification of the functions of many genes. In the near future, a similar approach using a genome-wide RNAi library should be feasible in mammalian cells because libraries of siRNA oligonucleotide and siRNA-expression vectors are becoming available. Randomized ribozyme libraries have already been used for the identification of novel genes [53–68] and several groups have begun to generate siRNA libraries directed against the entire human genome [69–79].

4. Approaches to the construction of RNAi libraries

The successes achieved by the exploitation of RNAi for the analysis of the functions of individual genes and by use of ribozyme libraries have led inevitably to efforts to apply these approaches on a large scale to reverse genetic analysis. In the case of construction of an RNAi library, the library can consist of either synthetic siRNAs or siRNA-expression vectors. Plasmids and viral vectors are exploitable expression systems for the siRNA-expression library. Each library has the advantages and disadvantages in its delivery strategies. For example, although viral vectors can be imported efficiently into various cell lines by infection, siRNAs and plasmid vectors cannot be imported efficiently into some cell lines, such as primary, neural and hemopoietic cells, by transfection. Transfection of siRNAs and plasmid vectors can be performed easily in ordinary laboratories, but handlings of viral vectors require more experiences and special equipments. It is necessary to choose the delivery strategy that is optimal under a fixed experimental condition.

Genome-wide libraries of siRNAs can be constructed in several fundamentally different ways, which include chemical synthesis and the enzymatic digestion of long dsRNAs. Because the effectiveness of siRNAs is strongly dependent on their target sites in their target RNAs, several groups developed their own algorithm that allows one to predict favorable target sites for generation of a high-quality library [11–18,80–90]. Some of these algorithms are open to the public on the web. In our case, in order to optimize various parameters, we decided to produce a large number of siRNA pairs and measure sequence–activity relationships by walking along an entire gene and shifting one base at a time [41]. Unfortunately, in the year 2001, the price of one siRNA pair in Japan was close to $1000! However, Suzuki's group has since developed a transcription system that can be used to produce siRNAs at a reasonable price [91] (Fig. 2). The advantage of their strategy is that only completely base-paired siRNAs are collected from the gel and, thus, the concentration of oligonucleotides reflects that of duplexed siRNAs. Examination of the sequences of approximately 1000 siRNA pairs directed against the mRNA for EGFP and their activities enabled us to develop an original and reliable algorithm [16,92,93].

Prior to the construction of the siRNA-expression library, we also optimized our siRNA-expression system. We constructed three types of siRNA vector system, namely, a tandem type, a dual-promoter type, and a hairpin type, and compared the activities of the resultant siRNAs. We determined that the hairpin-type system had the highest suppressive activity at the low concentrations of plasmids. However, we observed a high rate of mutation in the stem-loop region when we used plasmids to transform *Escherichia coli*. These mutations presented a serious problem with respect to the construction of a reliable library of siRNA vectors. However, we found that introduction of multiple C to T (or A to G) mutations into the sense strand rendered the plasmids genetically stable and did not affect silencing activity (Fig. 3). Moreover, we optimized the loop sequence that connected the sense and antisense sequences and other parameters that might be expected to affect the activity of the siRNA. Together, the optimization of the siRNA-expression system and the development of our algorithm enabled us to construct a large-scale and high-quality library of siRNA vectors. Now, using our algorithm and our

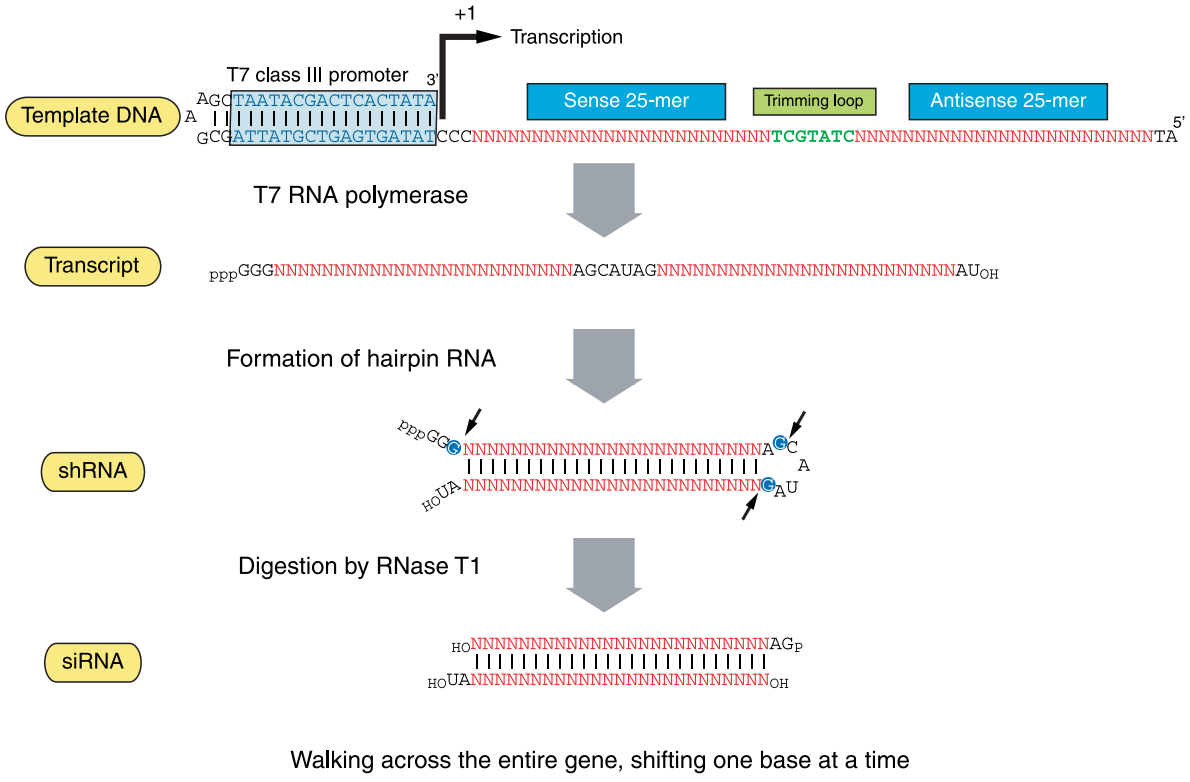

Fig. 2. Schematic representation of a transcription system for production of siRNA.

optimized siRNA-expression system, we are constructing an siRNA-expression library that will encompass the complete array of transcripts from the human genome (Fig. 3). To confirm that the same phenotypic changes occur when siRNAs are targeted to multiple independent sites in an mRNA, our library contains at least two target sites per gene [79].

Although our libraries are based on the optimized algorithm for selection of the best two target sites, it might also be possible and more economical to use enzymatically fragmented cDNAs for the construction of libraries. Indeed, several groups have reported methods for generating libraries of random shRNAs using fragments of enzymatically digested cDNA [94–96]. Sen et al. described an enzyme-mediated method for generating numerous functional siRNA constructs from any gene of interest or any pool of genes. The restriction enzyme-generated siRNA (REGS) system produced, on average, 34 unique siRNAs per kilobase of sequence. REGS enabled Sen et al. to create a complex siRNA library from double-stranded cDNA that encompassed known and unknown genes, with 96% of the clones containing inserts of the appropriate size [94].

Shirane et al. produced RNAi libraries from cDNAs using another system that they called EPRIL (enzymatic production of RNAi library). EPRIL involves several enzymatic treatments for the production of a library of shRNA-expression vectors from cDNAs of interest and should contribute significantly to the elucidation of the gene function at the whole-genome level [95].

Luo et al. described a system for "siRNA production by enzymatic engineering of DNA (SPEED)". Theirs is a simple, effective, and inexpensive strategy for construction of genome-wide siRNA-expression libraries from populations of double-stranded cDNAs. The method dispenses with the need for expensive chemical synthesis of oligonucleotides and facilitates the generation of siRNA libraries that encompass all expressed genes, including those of unknown structure and function, from diverse sources of mRNA in many species. In addition to cDNA libraries, other DNAs can be converted into an siRNA library by SPEED, for example, the HIV genome. Screening of such a library for siRNAs that strongly inhibit viral replication or other virus-encoded functions should help to identify potential siRNA-based therapeutic agents [96].

One potential drawback associated with this kind of cloning of enzymatically fragmented short DNAs is the possible production of fragments of palindromic DNA (which lead to formation of completely matched hairpin RNAs) that tend to mutate during amplification in *E. coli*, as described above. The maintenance of reliable libraries requires special care. Thus, we shall have to wait to assess the utility of such libraries for the successful identification of functional genes.

5. Application of RNAi libraries

We have used our library to screen for genes involved in the endoplasmic reticulum stress-dependent apoptosis that is induced by thapsigargin, a plant-derived sesquiterpene lactone [77]. Thapsigargin triggers endoplasmic reticulum stress, with subsequent apoptosis, but the molecular mechanisms underlying this process are incompletely understood. Using the library, we were able to identify some unexpected and novel pathways in thapsigargin-induced apoptosis, and our results provide evidence for the efficacy and utility of the comprehensive analysis of signaling networks and pathways with a library

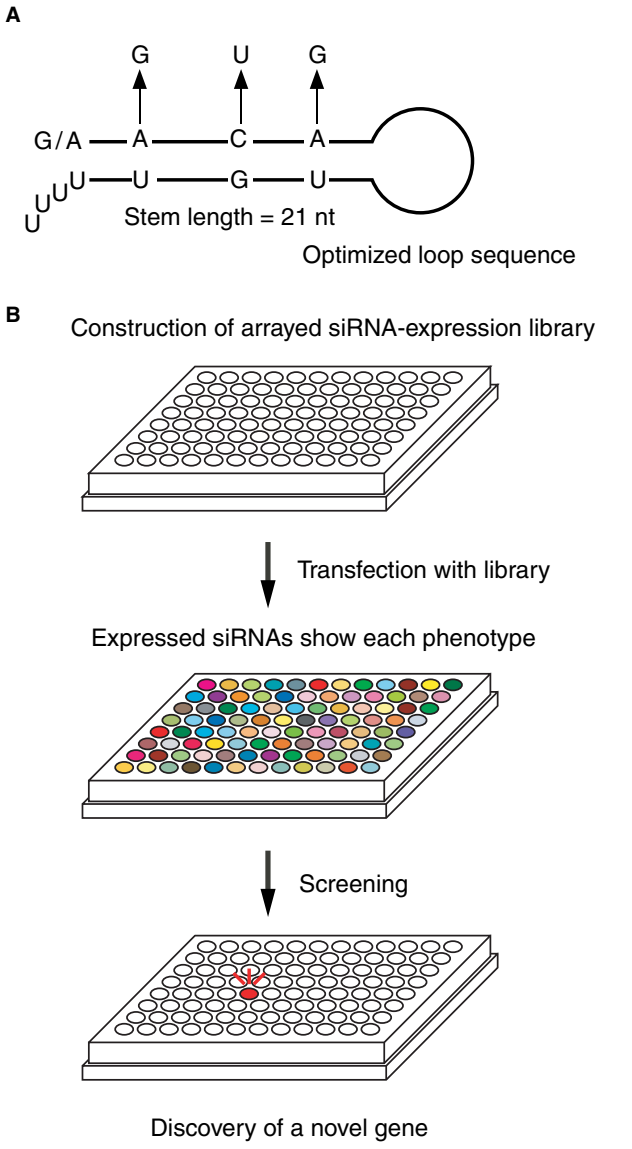

Fig. 3. (A) Schematic representation of the proposed siRNA-expression system. Three or four C to U or A to G mutations are introduced into the sense strand. (B) Schematic representation of the discovery of a novel gene using an siRNA library.

of siRNA-expression vectors. The most significant advantages of our siRNA-expression library, as compared with the widely used library of synthetic siRNAs are as follows. The interferon response in transfected cells can be avoided [28,29], and the RNAi effect is sustained for a longer period of time than are the inhibitory effects of synthetic siRNAs, in particular, in proliferating cells. Using the siRNA-expression library, we demonstrated the straightforward identification of novel pathways that had not previously been identified by other strategies [66,67,75,77,79]. We have already constructed siRNA libraries directed against the genes for all human kinases and phosphatases and against the genes for all human and mouse transcriptional factors and nuclear factors, as well as against other human genes (~15000 clones). Further analyses using such large libraries of siRNA-expression vectors should provide more precise information about various signaling pathways and enhance our understanding of numerous physiological phenomena. As a consequence, they should allow us to identify specific disease-related target genes.

Many groups have used either libraries of synthetic siRNAs or of siRNA-expression vectors that had been produced by the selection of specific cleavage sites within target mRNAs [69–74,78]. Some groups have reported the application of libraries of synthetic siRNAs directed against certain families of genes [70,72,78]. Aza-Blanc et al. screened HeLa cells using an siRNA library directed against 510 genes, including the genes for most human kinases, to identify genes that impact TRAIL-induced apoptosis. TRAIL is a member of the tumor necrosis factor (TNF) superfamily that induces the selective killing of tumor cells when bound to its cognate receptor. Using this approach, Aza-Blanc et al. [70] identified a variety of known and previously uncharacterized genes that modulate TRIAL activity.

Mackeigan et al. systematically screened the kinase- and phosphatase-encoding components of the human genome and identified new regulators of apoptosis and chemoresistance using a custom-made set of libraries of siRNAs that were designed to include two siRNA duplexes for each gene target. In total, their kinase library was directed against 650 target genes and their phosphatase library was directed against 222 genes. The development of inhibitors that target these kinases or phosphatases may lead to new anti-cancer strategies [78].

Zheng et al. reported the development of a dual-promoter siRNA-expression system. In this system, a gene-specific siRNA sequence is inserted between two different opposing pol III promoters, the mouse U6 and the human H1 promoters. They used their system to construct a library of siRNA-expression cassettes that targets 8000 human genes with two designed sequences per gene [71]. They were able to identifiy both known and unique regulators of NF-κB signaling using this library.

Brummelkamp et al. reported the production of an shRNA library directed against the family of de-ubiquinating enzymes [69]. Protein modification via the conjugation of ubiquitin moieties, known as ubiquitination, plays a major role in many biological processes, including the cell cycle and apoptosis. The enzymes that mediate ubiquitination have been well studied, but much less is known about the ubiquitin-specific proteases that mediate de-ubiquitination of cellular substrates. To study this family of proteins, they designed shRNA vectors for suppression of the expression of 50 human de-ubiquitinating enzymes, and used these vectors to identify de-ubiquitinating enzymes in cancer-relevant pathways. Using the library, they identified the tumor suppressor CYLD (encoded by the familial cylindromatosis-susceptibility gene) as a suppressor of NF-κB activity. CYLD binds to the NEMO (also known as IKKg) component of the IkB kinase (IKK) complex and appears to regulate its activity through de-ubiquitination of TRAF2, since the ubiquitination of TRAF2 can be modulated by CYLD. Inhibition of CYLD increases resistance to apoptosis, suggesting a mechanism through which loss of CYLD might contribute to oncogenesis. This effect can be relieved by aspirin derivatives that inhibit NF-κB activity, an observation that suggests a therapeutic intervention for restoration of growth control in patients with familial cylindromatosis. These results led to proposals for treating cylindromatosis with existing drugs and provided powerful confirmation that unbiased,

genetic approaches can lead not only to new insights in biology but also to practical advances in the treatment of disease.

Two independent groups have recently reported the usefulness of viral libraries with bar code detection [73,74]. Berns et al. reported the construction of a set of retroviral vectors that encode 23 742 distinct shRNAs and target 7914 different human genes [73]. They used their library in human cells to identify one known and five previously unknown modulators of arrest of the p53-dependent proliferation. Suppression of these genes conferred resistance to the arrest of both the p53-dependent and the p19ARF-dependent proliferation of cells, and abolished the DNA damage-induced arrest of the cell cycle at G1 [73]. Paddison et al. reported the construction and application of an shRNA-expression library that targeted 9610 human and 5563 mouse genes. Their library consists of close to 28 000 sequence-verified shRNA-expression cassettes contained within multifunctional vectors, which permit the shRNA cassettes to be packaged in retroviruses, tracked in mixed populations of cells by means of DNA 'bar codes', and shuttled to customized vectors by bacterial mating. In order to validate the library, Paddison et al. used a genetic screen designed to identify defects in human proteasome function. Their results suggest that large-scale RNAi libraries can be used in specific, genetic applications in mammals and will become valuable resources for gene analysis and the discovery of potential drug targets [74,97].

Genome-wide RNAi screenings can contribute to the rational design of drugs against heritable, infectious and malignant diseases. For example, Eggert et al. performed genome wide RNAi screening combined with small molecule screening [98]. Their parallel chemical genetic and genome wide RNAi screens identified cytokinesis inhibitors and targets and showed that the parallel RNA interference with small molecule screening is useful approach to discover leads for therapeutic drugs. In addition, Agaisse et al. performed genome-wide RNAi screen for host factors required for intracellular bacterial infection [99].

Now, a number of libraries are commercially available, for example the MISSION shRNA library from Sigma Aldrich, the Hannon-Elledge shRNA library from Open Bio Systems, the *s*mart & *i*ntelligent *si*RNA library from iGENE therapeutics, etc. These libraries should be useful for discovery of potential drug targets.

6. Conclusion

The available methods for construction of RNAi libraries each has various advantages and defects. Random libraries are relatively inexpensive to produce and can cover an individual gene with many different shRNAs. However, they do not necessarily cover all genes and the vector might not be stable. By contrast, libraries produced from chemically synthesized siRNAs are easy to handle and transfection efficiencies are generally high, but they are expensive and the duration of their efficacy is limited. Structurally optimized effective shRNAs, transcribed from vectors in cells, can now be generated by applying an optimized algorithm and they can be maintained with ease. In future, siRNA libraries will be useful for wide-range screening for drug development. There is no question that these libraries and large-scale RNAi libraries will contribute to our understanding of numerous biological phenomena and to the design of entire classes of novel therapeutic agents.

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